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Delayed labelling of brain glutamate after an intra-arterial [^{13}C]glucose bolus: evidence for aerobic metabolism of guinea pig brain glycogen store

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Abstract

Glycogen in glial cells is the largest store of glucose equivalents in the brain. Here we describe evidence that brain glycogen contributes to aerobic energy metabolism of the guinea pig brain in vivo. Five min after an intra-arterial bolus injection of D-[U-^{14}C]glucose, 28 ± 11% of the radioactivity in brain tissue was associated with the glycogen fraction, indicating that a significant proportion of labelled glucose taken up by the brain is converted to glycogen shortly after bolus infusion. Incorporation of ^{13}C-label into lactate generated by brains made ischaemic after D-[1-^{13}C]glucose injection confirms that these glucose equivalents can be mobilised for anaerobic glucose metabolism. Aerobic metabolism was monitored by following the time course of ^{13}C-incorporation into glutamate in guinea pig cortex and cerebellum in vivo. After an intra-arterial bolus injection of D-[1-^{13}C]glucose, glutamate labelling reached a maximum 40–60 min after injection, suggesting that a slowly metabolised pool of labelled glucose equivalents was present. As the concentration of ^{13}C-labelled glucose in blood was shown to decrease below detectable levels within 5 min of bolus injection, this late phase of glutamate labelling must occur with mobilisation of a brain storage pool of labelled glucose equivalents. We interpret this as evidence that glucose equivalents in glycogen may contribute to energy metabolism in the aerobic guinea pig brain.

Keywords: Glycogen; Glutamate; Ischemia; Metabolism; ^{13}C-NMR spectroscopy

1. Introduction

Glycogen (0.6–1.6 mg/g wet wt. in the guinea pig brain[1]) is the single biggest carbohydrate energy store in the brain [2], with the majority of glycogen found in astrocytes [3]. Metabolism of glucose equivalents of glycogen for lactate synthesis during ischaemia and hypoxia is well-known [4]. However, brain glycogen concentration also varies with synaptic activity, e.g. it increases during barbiturate-induced anaesthesia [5]. Swanson [6] has shown that there is direct coupling of glial glycogen metabolism to neuronal activity, and release of specific neurotransmitters promotes glycogenolysis [7]. Although a traditional view has been that glycogen is metabolically inactive under normal conditions in vivo, radiotracer studies suggest there is substantial turnover of labelled glycogen in the well-perfused, normoxic state

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[1]. Poitry-Yamate and colleagues [8] suggested that Müller cells found in the guinea pig retina export glycogen-derived lactate to neurones to support energy metabolism.

In the present study, the labelling of metabolites was followed in the guinea pig brain using isotope-labelled glucose as a tracer compound. Relative glucose flux into the brain glycogen fraction was tested and then label incorporation into lactate was used as an index of anaerobic metabolism of glucose equivalents in glycogen. In order to test the hypothesis that brain glycogen also contributes to the energy metabolism of the well-perfused guinea pig brain in vivo, the distribution of ^{13}C-label in glutamate was followed with ^{13}C- and ^{1}H- high resolution NMR after intra-arterial infusion of D-[1-^{13}C]glucose.

2. Materials and methods

2.1. Materials

D-[1-^{13}C]glucose (99.9%), D-[U-^{14}C]glucose and [^{13}C]formate (99%) were purchased from Cambridge Isotope Laboratories, Andover, MA, USA. Optisolve was obtained from LKB Scintillation Products (Loughborough, UK). Cytoscint scintillation fluid was purchased from ICN (Costa Mesa, CA, USA). All other reagents were of AR grade.

2.2. Methods

2.2.1. Metabolism of D-[1-^{13}C]glucose by guinea pig cortex and cerebellum in vivo

All experiments were conducted within the welfare for animals guidelines of the United Kingdom Home Office.

Guinea pigs (Hartley), fed ad libitum prior to the procedure, were anaesthetised with fentanyl/fluanisone (Hypnorm) and midazolam (Hypnovel) by intramuscular injection (0.2 ml/kg of Hypnorm followed 10 min later with 1 mg/kg of Hypnovel) and a cannula was inserted into the left carotid artery. The cannula was inserted upstream (i.e. in the direction of the heart). The vascular anatomy is such that fluid injected upstream into the left carotid artery enters the brachiocephalic trunk and is then forced to enter the right carotid artery or the right subclavian artery. This method was adopted due to the tendency of the delicate guinea pig artery to collapse when high concentrations of glucose were injected in a downstream direction.

Blood glucose both before and after the administration of the bolus of glucose was measured using an ExacTech blood glucose pen (MediSense, Waltham, USA). The duration of the injection of the bolus was less than 10 s. A toe-nail was clipped to provide the blood sample for blood glucose testing. Resting blood glucose levels for the guinea pig were found to be 3.7 ± 0.5 mM and had reached a level of 6.0 ± 0.9 mM 5 min after the administration of the bolus. At subsequent time points, the blood glucose was found to lie in the range 3.8 ± 0.8 mM.

To study metabolism in the presence of anaesthesia, a solution of D-[1-^{13}C]glucose in physiological saline (20% w/v, 0.15 mg/g b.wt.) was infused within 10 s. The guinea pig was sacrificed by cervical dislocation at either 5, 10, 20, 30, 40 or 60 min post-infusion ($n = 3$), and the cortex and cerebellum rapidly dissected out and frozen in liquid nitrogen (less than 15 s from death to complete freezing). For the awake animal study ($n = 3$, except at 30 and 40 min where $n = 4$), the cannula was passed beneath the skin to emerge at the back of the neck and the wound sutured during anaesthesia. Systemic analgesia was used to minimise discomfort (45 μg/kg buprenorphine).

The animal was allowed to recover from anaesthesia for at least 18 h. During recovery, topical lignocaine was used as a local anaesthetic to minimise discomfort at the wound site. A solution of D-[1-^{13}C]glucose was then infused into the cannula as above. Frozen tissue samples were extracted, neutralised and lyophilised as above. Protein estimations were performed on the pellet obtained after tissue extraction with perchloric acid using the method of Miller [9].

Following sacrifice, 5 ml of blood was removed and immediately placed in an equal volume of perchloric acid, extracted and freeze-dried as above. No residual ^{13}C-label was detected in ^{13}C-NMR spectra of blood extracts from any time point. On some occasions, the liver was also dissected, freeze-clamped and extracted. Only trace amounts of ^{13}C-label were detected in ^{13}C-NMR spectra obtained of liver extracts.

2.2.2. Metabolism of D-[U-^{14}C]glucose by guinea pig cortex and cerebellum in vivo

To investigate the fate of the bolus of glucose (as NMR detection of ^{13}C is less sensitive compared with radioisotope detection of ^{14}C), infusions of D-[U-^{14}C]glucose were carried out in three anaesthetised guinea pigs in an identical manner to those described above. A bolus of solution containing D-glucose (20% w/v, 0.15 mg/g b.wt.) labelled with 20 μCi of D-[U-^{14}C]glucose in physiological saline was infused. The animals were sacrificed after 20 min by cervical dislocation and the brain and 5 ml of blood were removed. Tissue was dissolved in Optisolve (200 mg of tissue to 1 ml of optisolve) at 60°C, diluted four-fold in Cytoscint and radioactivity was measured using a LS 1701 Scintillation Counter (Beckman, UK).

To investigate whether glycogen was labelled after an infused bolus of D-[U-^{14}C]glucose, animals were anaesthetised and cannulated as above ($n=6$). The skin above the skull was removed, and a bolus containing D-glucose (20% w/v 0.15 mg/g b.wt.) labelled with 20 μCi of D-[U-^{14}C]glucose in physiological saline was administered within 10 s. Five minutes after the administration of the bolus, the brain was frozen by pouring liquid nitrogen through a funnel onto the skull. The brain was removed and sections dissolved in KOH (40 mg of tissue to 0.5 ml of 1 M KOH) by heating for 20 min at 70°C. Samples of the dissolved tissue were counted for radioactivity in the manner described above. Glycogen was precipitated from other tissue samples dissolved in KOH using ice-cold ethanol by the method described by Stauffacher and Renold [10] and Cuendet and co-workers [11]. Precipitated glycogen was redissolved in water and reprecipitated in ice-cold ethanol to ensure that glucose and glucose-6-phosphate were removed from the fraction. The fraction may, however, still have contained some amount of long chain polysaccharide carbohydrates other than glycogen. The pellet was dissolved in water and the radioactivity of the sample was examined in the manner described above. However, while we have found that this procedure gives acceptably precise measurements, we suspect that it may underestimate the total labelled glycogen content in brain. The assay was developed as a general method for glycogen estimation in tissues and optimised for skeletal muscle, in which glycogen may be more stable during the extraction than in brain.

Label incorporation is expressed as a percentage of the injected radioactivity per gram wet weight of tissue.

2.2.3. Acquisition of NMR spectra

Lyophilised samples were resuspended in D_2O containing 4 mM [^{13}C]formate as an internal intensity and chemical shift reference and dispensed into 5 mm NMR tubes. All ^{1}H-NMR spectra were acquired at 400.12 MHz on a Varian INOVA spectrometer (Varian Associates, Palo Alto, CA) across 32K data points using a repetition period of 30 s for 'fully relaxed' spectra. Free-induction decays were transformed using zero-filling from 32K to 64K data points and an exponential multiplication factor of 0.5 Hz. Peak assignment was aided by reference to standard spectra of authentic compounds.

All ^{13}C-NMR spectra were acquired at 100.61 MHz across 128K data points using a repetition period of 3.485 s with broad-band proton decoupling (WALTZ-16 [12]) during the acquisition time. Free-induction decays were transformed using zero-filling from 128K to 256K data points and an exponential multiplication factor of 3 Hz. Peak assignment was aided by reference to standard spectra of authentic compounds. Peak areas were determined using standard Varian software (version 5.2f). The concentration of isotopomers was calculated by multiplying peak areas by a saturation factor obtained from fully relaxed spectra which allowed for spin-relaxation and nuclear Overhauser effect (n.O.e), and comparing this with the area of an internal concentration standard. The combined effect of spin-relaxation and n.O.e differed markedly for the three resonances of glutamate with the pulse sequence used. Thus, a direct comparison of intensities of glutamate resonances cannot be performed without use of correction factors Spectra were also obtained of tissue extracts in the absence of any ^{13}C-label in order to compensate for natural abundance contributions. Metabolite enrichment is expressed as an absolute measure of micromoles ^{13}C-label incorporated per gram wet weight of tissue extracted.

2.2.4. Estimation of metabolic pool sizes

The concentrations of glutamate, glutamine and

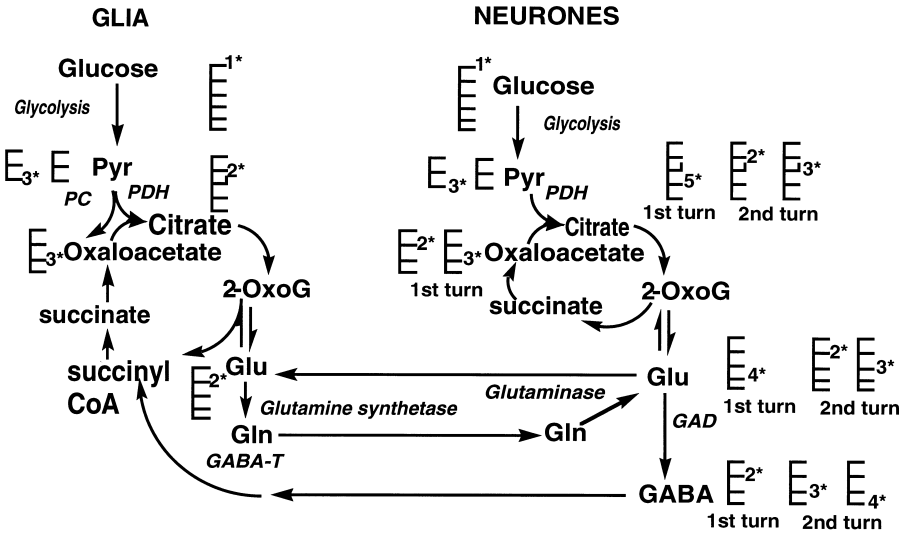

Fig. 1. The labelling pattern of metabolites by ^{13}C from the metabolism of D-[1-^{13}C]glucose depends on the pathways involved. Glucose is metabolised by glycolysis to produce an equal quantity of unlabelled and [3-^{13}C]pyruvate. Subsequent metabolites are labelled at different positions depending on whether [3-^{13}C]pyruvate is a substrate for pyruvate dehydrogenase or the glial-only enzyme pyruvate carboxylase, as well as how many turns of the TCA cycle occur before the metabolite is labelled. Considering glutamate and glutamine, the carbon backbone is labelled at the C4 position on the first turn of the cycle via pyruvate dehydrogenase and then at the C2 and C3 position with equal probability on the second turn. The C2 position is also labelled via pyruvate carboxylase. Thus, the labelling pattern is indicative of the relative rate of activity of the enzymes pyruvate carboxylase and pyruvate dehydrogenase. For simplicity, only the pyruvate carboxylase pathway products are shown for glia. Key: PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; 2-Oxoglu, 2-oxoglutarate; OAA, oxaloacetate; Glu, glutamate; Gln, glutamine. * ^{13}C-label.

lactate in cortex and cerebellum extracts were estimated for each time point using ^{1}H-NMR. Peak areas were determined from fully relaxed spectra for glutamate (γ-C$\underline{H}_2$), glutamine (γ-C$\underline{H}_2$) and lactate (C$\underline{H}_3$) and compared with the reference peaks of formate ($n=12$; two for each time point).

2.2.5. *Analysis of the labelling of metabolites by D-[1-^{13}C]glucose*

During the metabolism of D-[1-^{13}C]glucose glutamate and glutamine are labelled at different positions depending on the pathway involved (Fig. 1). Pyruvate dehydrogenase is found in both glia and neurones. Glutamate and glutamine derived from label entering the tricarboxylic acid (TCA) cycle via this enzyme will be labelled at the C4 position on the first turn of the cycle and then with equal probability at the C2 and C3 positions on the second turn. However, glutamate and glutamine derived from label entering the tricarboxylic acid cycle via the glial-only enzyme pyruvate carboxylase are labelled at the C2 position. Thus, the quantity of glutamate derived via anaplerosis (pyruvate carboxylase pathway) on the first turn of the cycle is given by the difference between C2 and C3 labelling of glutamate provided only two turns of the TCA cycle are considered. Shank and co-workers [13] defined the anaplerotic ratio (R) for glutamate, an approximate measure of the contribution of anaplerosis compared to the catabolic pathway in the amino acid's production as

$$R = ([C2\ glu]-[C3\ glu])/[C4\ glu] \quad (1)$$

where [Cx glu] indicates the concentration of Cx-labelled glutamate. However, R is time-dependent and becomes a less accurate measure (underestimating the true proportion of anaplerosis) of the amount of glutamate produced by anaplerosis at longer times after labelling.

2.2.6. *Statistical analysis*

Data are expressed as mean ± S.D. Statistical analysis was performed using Student's unpaired, two-tailed t-test. Differences in mean values were consid-

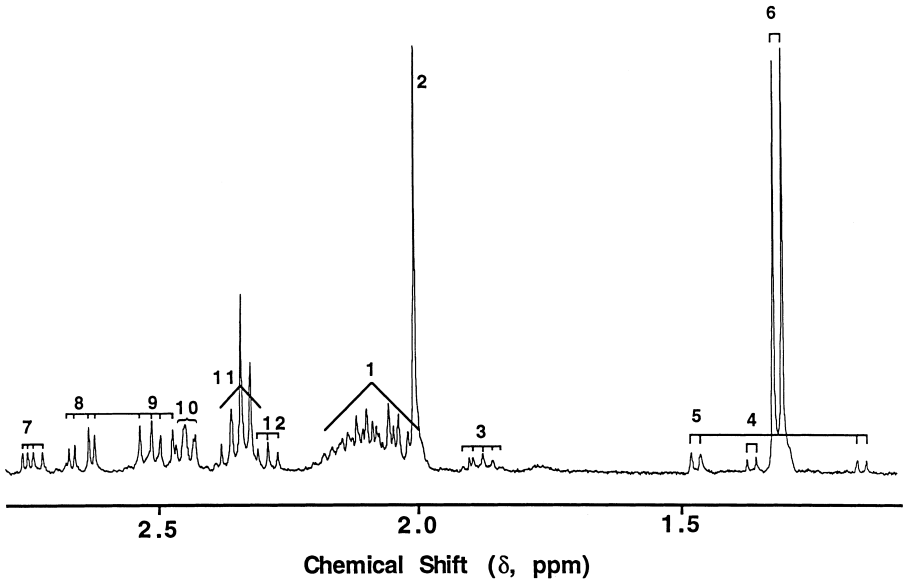

Fig. 2. Fully relaxed 400.12 MHz ^{1}H-spectrum of an extract from an awake guinea pig cortex following 40 min of metabolism of D-[1-^{13}C]glucose. The resonances of γ-glutamine, γ-glutamate and CH_3 lactate were used to calculate the total extractable amount of metabolite found in the extract. The ^{13}C-sideband resonances for CH_3 lactate were used to calculate the total concentration of [3-^{13}C]lactate detected and cross checked with calculations from ^{13}C-spectra. Key to ^{1}H-resonances: 1, β/β′-glutamate/glutamine; 2, CH_3 *N*-acetyl aspartate; 3, γ-GABA; 4, β-alanine; 5, ^{13}C-sidebands of CH_3 lactate; 6, CH_3 lactate; 7, β/β′-aspartate; 8, β′-*N*-acetyl aspartate/aspartate; 9, β-*N*-acetyl-aspartate/aspartate; 10, γ-glutamine; 11, γ-glutamate; 12, β-GABA; p.p.m., parts per million.

ered statistically significant at $P < 0.05$. For the awake brain an ANOVA test analysis was performed, with a test for non-linearity using InStat for Macintosh (GraphPad software) to judge whether any time points were considered significantly different from the trend detected in ^{13}C-labelling.

3. Results

3.1. Brain glycogen incorporates labelled glucose after an intra-arterial bolus injection

To investigate whether a bolus injection of D-[U-^{14}C]glucose is incorporated into brain glycogen, the relative incorporation of D-[U-^{14}C]glucose into the brain homogenate glycogen fraction was measured. To limit ischaemic production of lactate from glycogen during tissue extraction, funnel freezing of the brain was used. Five min after intra-arterial injection of a bolus of glucose containing trace amounts of D-[U-^{14}C]glucose, $28 \pm 11\%$ ($n = 6$) of the label in the brain was found in the glycogen fraction.

3.2. Labelled glucose is cleared rapidly from the blood after bolus injection

The time course of ^{14}C-labelled glucose in the blood after intra-arterial injection of a glucose bolus (0.15 mg of glucose/g b.wt.) with trace amounts of D-[U-^{14}C]glucose (20 μCi) was followed to define the distribution of label in the guinea pig. At 20 min after injection, only $1.03 \pm 0.03\%$ ($n = 3$) of the total injected label was still present in blood ($0.027 \pm 0.001\%$/g wet wt. of tissue, where wet weight has been calculated assuming 65 ml of blood/kg of guinea pig b.wt.). If all of the label was present in glucose (which must overestimate the labelled glucose as metabolites of glucose such as lactate also are present in the blood), then the enrichment of the blood with label at 20 min would be 0.035 at maximum (assuming normal plasma glucose concentration (3.7 mM)). Such a low specific activity of serum glucose could give a maximum enrichment of brain lactate and glutamate of only 0.018 ± 0.001.

A $1.3 \pm 0.2\%$ amount of the injected radioactivity was found in brain ($0.39 \pm 0.01\%$/g wet wt. of tissue). Assuming that 28% of this label was still associated

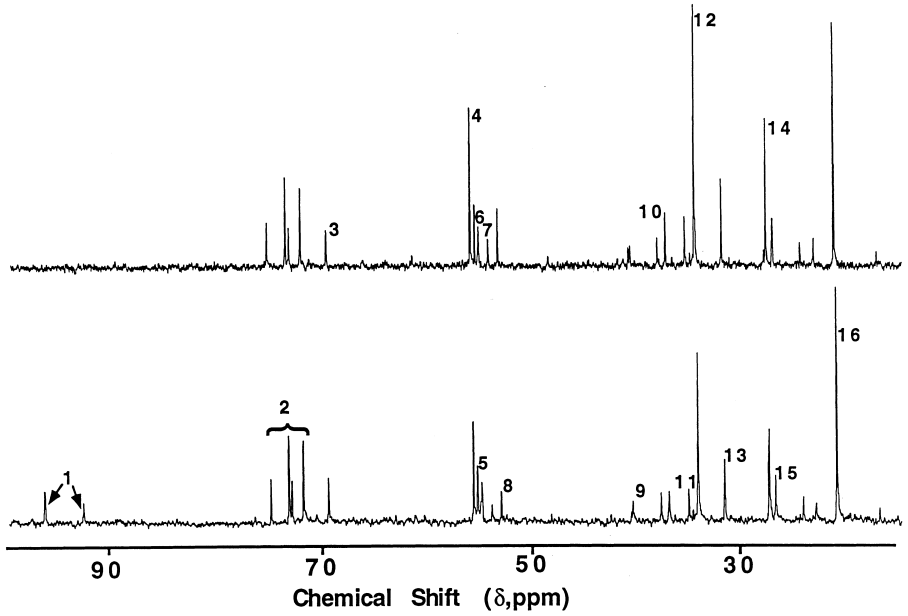

Fig. 3. ^{1}H-Decoupled ^{13}C-spectra of extracts from awake cortex (A) and cerebellum (B) after 30 min of metabolism of D-[1-^{13}C]glucose. Note that occasionally [1-^{13}C]glucose was detected. However, for any given sample, enrichment was less than 0.01 (assuming free glucose concentration ~1.6 μmol/g wet wt. [1]) and when averaged over all samples at no time point was the labelling significantly different from spectra obtained of brain tissue labelled by ^{13}C at natural abundance. The region from 10 to 100 ppm is shown. Resonances are relative to the internal standard [^{13}C]formate resonance at 172 ppm. Key (chemical shifts as parts per million (ppm) in parentheses): 1, C1 glucose (93.2 and 97.0 for α- and β-anomers); 2, C1–C6 inositol; 3, C2 lactate (69.6); 4, C2 glutamate (55.8); 5, C2 glutamine (55.4); 6, C2 *N*-acetyl aspartate (54.0); 7, C2 aspartate (53.4); 8, C2 alanine (51.7); 9, C2 GABA (40.1); 10, C3 aspartate (37.8); 11, C4 GABA (35.1); 12, C4 glutamate (34.6); 13, C4 glutamine (32.0); 14, C3 glutamate (28.3); 15, C3 glutamine (27.5), 16, C3 lactate (21.0).

with the glycogen fraction at 20 min (glycogen content was measured at 5 min post-infusion), infusion of a 90-mg bolus of glucose should produce ~0.33 mg or 1.8 μmol of labelled glucosyl units.

3.3. *Labelled glucose equivalents incorporated into glycogen are used for anaerobic metabolism during agonal ischaemia*

Brain extracts were prepared after a period of agonal ischaemia induced by decapitation. ^{13}C-NMR spectra were used to measure label incorporation into lactate. The C3 labelling of lactate reflects the prior label incorporation into glycogen, free glucose and lactate prior to death in the brain. The label incorporation into C3 lactate decreased with increasing time from the glucose bolus injection and to sacrifice. With decapitation at 5 min after bolus injection, C3 labelled lactate concentration was 0.60 ± 0.11 μmol/g wet wt. and 0.42 ± 0.13 μmol/g wet wt. for the cortex and cerebellum, respectively. In the cortex, labelling of lactate was reduced by 58 ± 12% with a 60-min delay between bolus injection and decapitation. Given the rapid decrease in blood glucose specific activity, the ^{13}C-label in lactate at 60 min must predominantly reflect label incorporation into glycogen.

3.4. *Glucose equivalents in glycogen are metabolised aerobically in the awake animal*

In order to determine the specific enrichment of individual metabolites with ^{13}C-label, the total concentrations of glutamate, glutamine and lactate were measured in rapidly frozen extracts using ^{1}H-NMR and label incorporation into metabolites was measured using ^{13}C-NMR (Figs. 2 and 3, Table 1). Labelling of the excitatory amino acid glutamate was used as a probe to monitor metabolism of

D-[1-^{13}C]glucose in the aerobic brain in vivo. After bolus injection of D-[1-^{13}C]glucose, the amino acids glutamate, aspartate and glutamine were labelled

readily in both the cortex and the cerebellum (Fig. 3). The dominant resonance was from C4 glutamate.

New label incorporation into glutamate occurred in the cortex and the cerebellum over more than 40 min. There was a trend suggesting an early phase of labelling with a maximum between 20 and 30 min with a relative glutamate C4 enrichment of 0.016 ± 0.003 and 0.026 ± 0.004 in the cortex and cerebellum, respectively (Fig. 4). The later labelling peak occurred between 40 and 60 min with an enrichment of 0.035 ± 0.04 and 0.050 ± 0.015 in glutamate in the cortex and cerebellum, respectively. The labelling at the C4 position of brain glutamate at 40–60 min was significantly greater than the linear trend for the first four time points ($F = 10.8$, $P < 0.001$, ANOVA), suggesting that there were two distinct phases of labelling. A similar time course of label incorporation was seen for the C2 and C3 glutamate positions.

In both the cerebral cortex and the cerebellum the anaplerotic ratio (Eq. 1 (a measure of the relative flux through glial-localised pyruvate carboxylase as opposed to pyruvate dehydrogenase) increased substantially after about 30 min following the bolus infusion (Fig. 5). By 60 min, $13 \pm 4\%$ and $33 \pm 6\%$ of the glutamate synthesis occurred via the anaplerotic pathway in the cortex and cerebellum, respectively.

Table 1
The total extractable concentration of certain metabolites found in cortical and cerebellar extracts in the awake and anaesthetised guinea pig

Metabolite concentration	Awake	Anaesthetised
Cortex		
Lactate	15.8 ± 4.6	10.5 ± 2.8[a]
Glutamate	13.0 ± 3.2	8.1 ± 2.7[b]
Glutamine	5.3 ± 2.1	4.3 ± 1.0
Cerebellum		
Lactate	15.9 ± 4.7	8.2 ± 2.3[c]
Glutamate	11.5 ± 4.1	8.2 ± 2.3
Glutamine	7.4 ± 3.5	2.4 ± 1.0[a]

Concentrations of these metabolites were not found to vary with time after the introduction of the bolus of D-[1-^{13}C]glucose and means are from averages of all time points (two for each time point; $n = 12$ unless stated). Values are mean ± S.D. μmol/100 mg of protein.
[a] $P < 0.05$
[b] $P < 0.005$
[c] $P < 0.001$ for significant difference between the awake and anaesthetised animal in either the cortex or the cerebellum, calculated using Student's unpaired t-test.

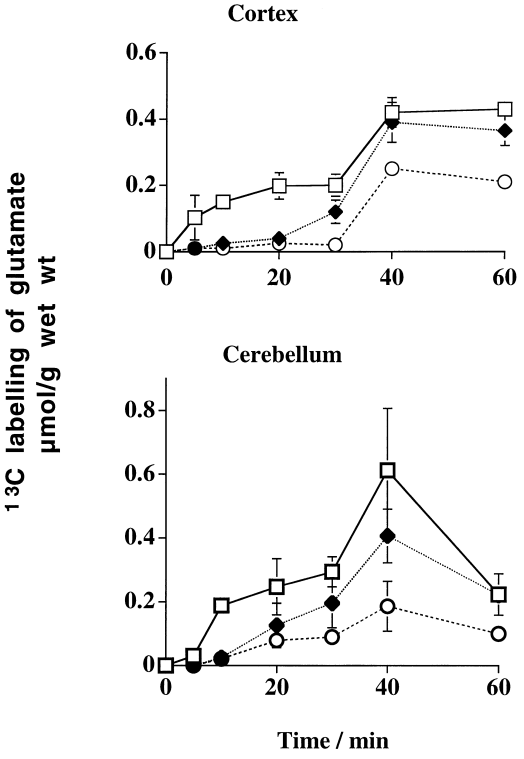

Fig. 4. The ^{13}C-labelling pattern of glutamate with time in the awake guinea pig cortex and cerebellum. Mean ± S.D. ($n = 3$, except for $t = 30$ min, 40 min where $n = 4$). In the cortex and cerebellum, the ^{13}C-labelling of glutamate was biphasic for the C2, C3 and C4 positions. C4 glutamate labelling dominated the labelling pattern. There was a significant difference between C2 and C3 labelling of glutamate after 40 min for the cortex and cerebellum, indicating substantial production of glutamate via pyruvate carboxylase. Key: q, C4 glutamate; u, C2 glutamate; m, C3 glutamate.

3.5. Anaesthesia delays labelling of C4 glutamate

The maximum C4 labelling in the cerebral cortex was achieved earlier in the awake than in the anaesthetised animal ($P = 0.03$ at 40 min) (Fig. 6).

3.6. Low glutamine enrichment demonstrates that the delayed labelling of glutamate does not arise from glutamine

To investigate the possibility that the delayed glutamate labelling may be due to new synthesis of glutamate from labelled glutamine, the time course of

enrichment and labelling pattern of glutamine was determined. In both the cortex and cerebellum, C4 glutamine was the most highly labelled glutamine resonance (Fig. 7). However, the relative enrichment of glutamine in both the cortex (maximum = 0.020 ± 0.007) and the cerebellum (maximum = 0.027 ± 0.008) of awake animals was always lower than that observed for glutamate, implying that a major fraction of the labelled glutamate must have been derived from an alternative pathway.

4. Discussion

Lowry and co-workers [4] observed that there is a rapid increase in lactate concentration post-mortem

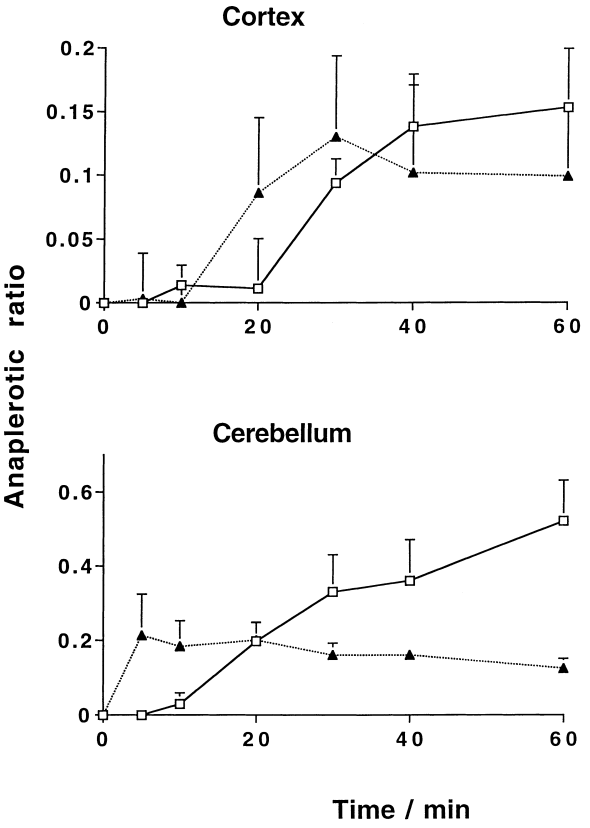

Fig. 5. The anaplerotic ratio for glutamate production in the cortex and cerebellum for the awake and anaesthetised guinea pig. Mean ± S.D. ($n = 3$, except for $t = 30$ min, 40 min where $n = 4$). The anaplerotic ratio measures the relative rate of labelling of a metabolite via the glial-only enzyme pyruvate carboxylase to that produced via pyruvate dehydrogenase. Key: q, awake animal; s, anaesthetised animal.

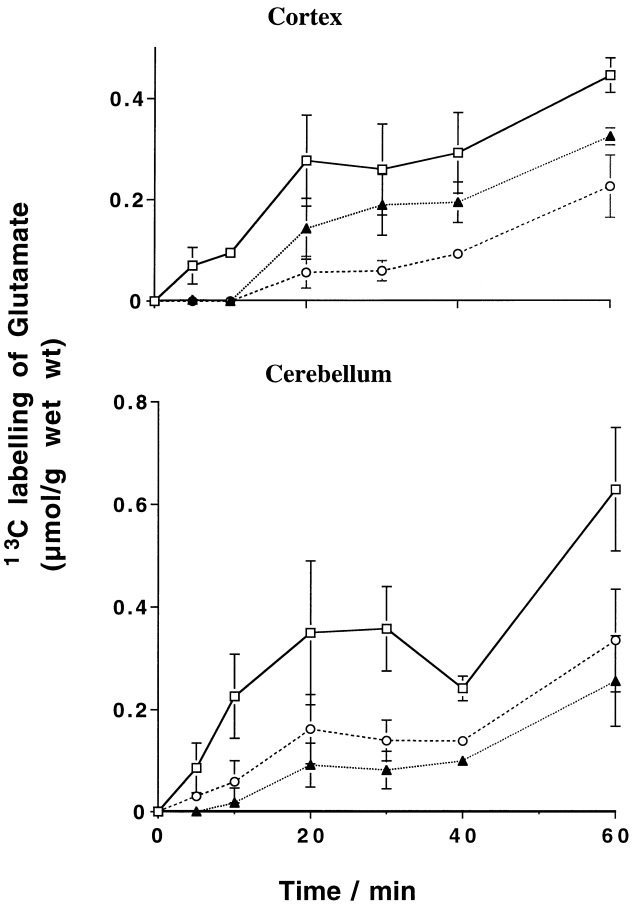

Fig. 6. The ^{13}C-labelling pattern of glutamate with time in the anaesthetised guinea pig for the cortex and cerebellum. Mean ± S.D. ($n = 3$). A biphasic labelling of glutamate occurred in the anaesthetised cerebellum, although unlike the awake animal, the second phase of labelling was delayed until 60 min. In the anaesthetised cortex, labelling of glutamate appeared to rise almost constantly with time. Key: q, C4 glutamate; s, C2 glutamate; m, C3 glutamate.

that is accompanied by a decrease in glycogen content of the brain. Here we also found that lactate concentrations in the cortical and cerebellar extracts prepared by decapitation from awake animals were 10-fold greater than values quoted for the normal range in vivo (0.8–2.0 mM [1]). Estimates of free glucose concentration [1] suggest that only 20% of the lactate detected could have arisen directly from brain glucose, suggesting that the majority of the lactate was derived from glycogen. As the specific enrichment of the lactate by ^{13}C-label was at least 2–3-fold greater than could be accounted for by the serum glucose enrichment, glucose equivalents in the

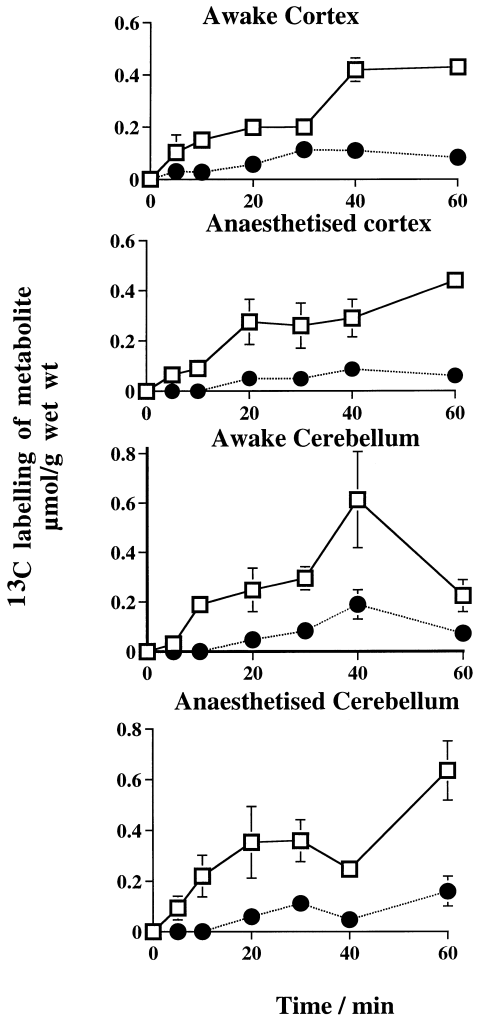

Fig. 7. The ^{13}C-labelling of glutamine at the C4 position compared with that of its precursor C4 glutamate. Mean ± S.D. ($n = 3$, except for $t = 30$ min, 40 min where $n = 4$ in the awake cortex and cerebellum). In both the awake and anaesthetised cerebellum, the labelling of glutamine and glutamate at the C4 position was very similar in pattern, showing biphasic labelling. However, in the cortex, no biphasic labelling of glutamine at the C4 position was observed in either the awake or the anaesthetised animal. Key: q, C4 glutamate; l, C4 glutamine.

brain glycogen pool labelled after the intra-arterial D-[1-^{13}C]glucose injection therefore must contribute substantially. This inference was supported by direct demonstration of incorporation of D-[U-^{14}C]glucose in the brain glycogen fraction after bolus intra-arterial injection.

Unlike lactate, glutamate concentrations and labelling in brain extracts accurately reflect concentrations in the brain in vivo [13,14] (both the TCA cycle and glutamate dehydrogenase are rapidly inhibited by the increases in NADH/NAD$^+$ ratio in the mitochondria that occur during hypoxia/ischaemia [15]). An earlier study of cerebral metabolism of intraperitoneally injected D-[1-^{13}C]glucose in rat brains failed to demonstrate a difference in glutamate and glutamine labelling between brain tissue left for 2 min or rapidly frozen [13]. Glutamate post-mortem appears to be remarkably stable, with Petroff and colleagues reporting only a small increase in brain pool size 1 h post-mortem in the rabbit [14].

Despite the rapid fall in serum labelled glucose, a significantly delayed time course for labelling of glutamate with ^{13}C at the C4 position was observed in both the cortex and cerebellum of the awake animal. Shank and colleagues [13] previously suggested that there may be a biphasic labelling pattern of cerebral glutamate following the injection of a bolus of D-[1-^{13}C]glucose in the rat, but they did not comment on its possible significance. Their results were consistent with rapid labelling over the first 5 min after bolus injection, followed by slower labelling to reach a maximum label incorporation after 45 min. In our study, the later phase of glutamate labelling was accompanied by an increase in the synthesis of glutamate by anaplerotic pathways, suggesting that there was a relative increase in contribution of metabolites in glial cells (as pyruvate carboxylase is compartmentalised in glia [16]).

The apparent biphasic time course for labelling of glutamate at the C4 position suggests that there may be at least two pools of D-[1-^{13}C]glucose equivalents. The pool contributing most to the earliest phase of labelling is likely to be derived directly from blood glucose. Previous work has demonstrated that labelled substrates introduced as a single bolus either subcutaneously or by intracarotid injection predominantly label the brain on the first circulation [17,18]. The labelling of glutamate in this early phase occurs with little anaplerosis, suggesting that the precursor labelled glucose equivalent is metabolised predominantly by neurones [13,19]. The significant delay between bolus injection and maximum C4 glutamate labelling in our experiments demonstrates that a proportion of the label is not directly incorporated into the TCA cycle immediately following the bolus. This suggests that there is an intermediate store of labelled

glucose equivalents that is released for metabolism more slowly.

The concentration and enrichment of free glucose in the brain is determined by the concentration and enrichment of plasma glucose [20,21]. We have shown directly that the specific enrichment of plasma glucose was too low to account for the later phase of glutamate labelling. This later phase of glutamate label incorporation also cannot be accounted for by synthesis from glutamine, which had a lower specific activity than did the glutamate.

Almost 30% of the bolus of labelled glucose isolated from the guinea pig brain was found in the glycogen fraction initially after the bolus injection. This suggests that ^{13}C-labelled glucose equivalents in glycogen are a potential precursor of the newly synthesised, labelled glutamate. The multi-chain, polymeric structure of glycogen (from which the most recently added glucose equivalents would be hydrolysed) would allow the specific activity of metabolites derived from glycogen soon after incorporation of new label could be highly enriched relative to the mean specific enrichment of the total glycogen pool. Thus, after a labelled glucose bolus, even a modest contribution of glucose equivalents from brain glycogen to synthesis of glutamate could enhance glutamate specific activity substantially.

Brain glycogen is predominantly found in glia [4,8]. The higher anaplerotic ratio for glutamate synthesised during the second phase of labelling is consistent with a higher contribution from glial-derived precursors to the total labelled glutamate precursor pool. There is substantial evidence that glial glycogen is metabolised to labelled lactate for export [22,23] and that it is glial-derived lactate that is used directly as a substrate for neurones [24–27]. This could, in part, explain the delay between bolus infusion and peak C4 glutamate labelling.

In contrast to our results obtained after bolus injection, a biphasic labelling pattern for glutamate was not observed in previous studies employing steady-state infusions of D-[1-^{13}C]glucose into the brain (e.g. [28,29]). However, as steady-state infusions of labelled substrate maintain a high, constant serum specific activity, such experiments should not be expected to be sensitive to the contribution from any less highly labelled intracellular pool. It also is possible that an intra-arterial bolus infusion of glucose may stimulate brain glycogen synthesis if there is a transiently high cytoplasmic free glucose concentration as a result of equilibration of cytoplasmic and serum glucose.

It has long been recognised that brain glycogen is a substrate for anaerobic glycolysis in the brain during hypoxia and ischaemia [3]. However, these experiments suggest that brain glycogen also may provide a dynamic store of glucose which can be in constant use in the well-perfused brain. There is a growing literature that glial glycogen content is affected by neuronal activity [8,30]. It is possible that provision of glucose equivalents from glycogen (rather than directly from glucose) is favoured kinetically during periods of higher metabolic rate (e.g. during functional activation) in a fashion similar to Ca^{2+}-induced mobilisation of glucose equivalents from glycogen in skeletal muscle during contraction [31]. Some of this may occur directly in neurones. However, the increase in the anaplerotic ratio for glutamate during the later phase of labelling is consistent with the notion that neuronal oxidative metabolism may rely, to a significant extent, on release of lactate generated from the metabolism of glial glycogen.

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